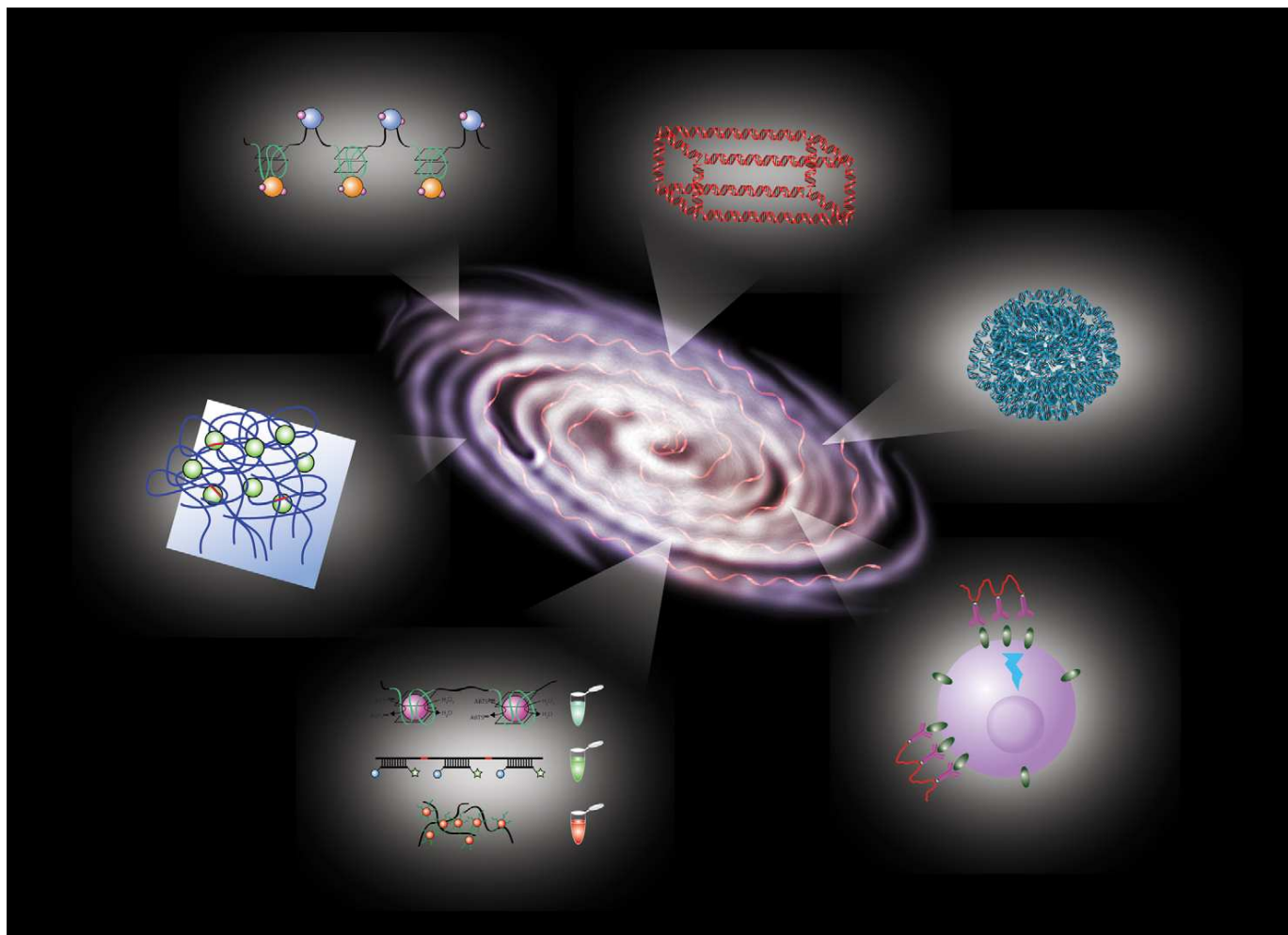


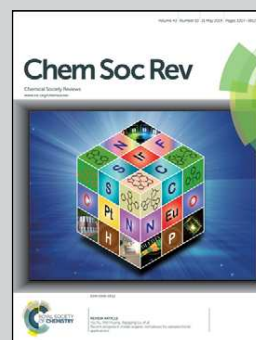


Featuring work from Dr Weian Zhao's group at University of California, Irvine whose research focuses on the development of bioengineering tools for disease diagnosis and regenerative medicine.

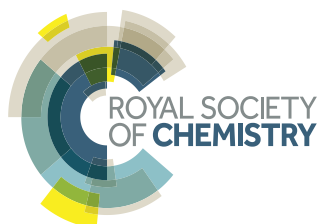
Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine

Rolling circle amplification (RCA) is an efficient and versatile isothermal enzymatic process to produce long nucleic acid products with defined sequences, valencies and structures. This review introduces the fundamental engineering principles of RCA, and discusses recent RCA-based bioanalytical tools, multivalent molecular scaffolds and nanostructures for applications in biology, diagnostics and therapeutics.

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Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine

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Rolling circle amplification (RCA) is an isothermal enzymatic process where a short DNA or RNA primer is amplified to form a long single stranded DNA or RNA using a circular DNA template and special DNA or RNA polymerases. The RCA product is a concatemer containing tens to hundreds of tandem repeats that are complementary to the circular template. The power, simplicity, and versatility of the DNA amplification technique have made it an attractive tool for biomedical research and nanobiotechnology. Traditionally, RCA has been used to develop sensitive diagnostic methods for a variety of targets including nucleic acids (DNA, RNA), small molecules, proteins, and cells. RCA has also attracted significant attention in the field of nanotechnology and nanobiotechnology. The RCA-produced long, single-stranded DNA with repeating units has been used as template for the periodic assembly of nanospecies. Moreover, since RCA products can be tailor-designed by manipulating the circular template, RCA has been employed to generate complex DNA nanostructures such as DNA origami, nanotubes, nanoribbons and DNA based metamaterials. These functional RCA based nanotechnologies have been utilized for biodetection, drug delivery, designing bioelectronic circuits and bioseparation. In this review, we introduce the fundamental engineering principles used to design RCA nanotechnologies, discuss recently developed RCA-based diagnostics and bioanalytical tools, and summarize the use of RCA to construct multivalent molecular scaffolds and nanostructures for applications in biology, diagnostics and therapeutics.

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1. Introduction

Rolling circle amplification (RCA) is a simple and efficient isothermal enzymatic process that utilizes unique DNA and RNA polymerases (Phi29, Bst, and Vent *exo*-DNA polymerase for DNA, and T7 RNA polymerase for RNA) to generate long single stranded DNA (ssDNA) and RNA (Fig. 1).^{1–6} In RCA, the polymerase continuously adds nucleotides (nt) to a primer annealed to a circular template which results in long ssDNA with tens to hundreds of tandem repeats (Fig. 1B). Unlike the polymerase chain reaction (PCR), which requires a thermal cyclor and thermostable DNA polymerases, RCA can be conducted

at a constant temperature (room temperature to 37 °C) in solution, on a solid support or in a complex biological environment (*e.g.*, on the cell surface or inside a cell). The ability of RCA to grow a long DNA chain on a solid support or inside a cell from one molecular binding event enables detection of targets at a single molecule level.^{7,8} Additionally, RCA products contain repetitive sequences that are complementary to the circular DNA template and therefore can be tailor-designed through manipulation of the template. By designing the template, the DNA products can be customized to include functional sequences including DNA aptamers,^{9,10} DNazymes,^{11–14} spacer domains,¹⁰ and restriction enzyme sites.^{15–17} Furthermore, multifunctional materials with diverse properties including biorecognition, sensing and imaging can easily be made by hybridizing RCA products with complementary oligonucleotides tethered with functional moieties including fluorophores, biotin, antibodies, and nanoparticles.^{18–21} Collectively, efficient isothermal amplification, single molecule sensitivity, structural and compositional versatility, and multivalency make RCA a powerful tool in biomedical and nanotechnology fields. Since its discovery in the mid 1990s, RCA has been explored extensively to develop sensitive detection methods for DNA,^{14,17,22–28} RNA,^{29–37} DNA methylation,^{38,39} single nucleotide

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polymorphism (SNP),^{40–49} small molecules,^{11,50,51} proteins,^{8,12,52–60} and cells.^{9,29,61,62} Besides diagnostics, RCA has also proven efficacious in cell-free cloning and sequencing,^{63–65} *in situ* genotyping,^{66,67} and whole-genome analysis in cells and tissues.^{42,65,68–70} More recently, RCA has garnered significant attention for its use in generating DNA nanostructures such as origami,^{2,71,72} nanoribbons,⁷² nanotubes,^{73–76} DNA nano-scaffolds for periodic nanoassembly,^{15,21,77–80} and DNA meta-materials.^{81,82} Importantly, these DNA based structures and materials hold great promise in a wide variety of applications including drug delivery, electronic circuit fabrication, and *in vivo* imaging. In this review we provide an overview of the fundamental engineering principles that enable RCA design. We then discuss recently developed RCA tools for diagnostics, biosensing and bioanalysis. Finally, we discuss recently

published RCA-based DNA nanostructures and materials for biological and therapeutic applications.

2. Fundamentals of RCA

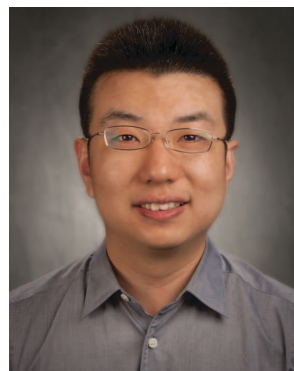
A typical RCA reaction requires four components: (1) DNA polymerase (*e.g.*, Phi29 DNA polymerase) including a suitable buffer (usually provided by manufacturer along with the polymerase); (2) a short DNA or RNA primer; (3) a circular DNA template; and (4) deoxynucleotide triphosphates (dNTPs) (monomers or building blocks of the RCA product). Among the polymerases, Phi29 DNA polymerase has been most frequently used due to its exceptional processivity and strand displacement ability. It has been shown that Phi29 can process



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Feng Li

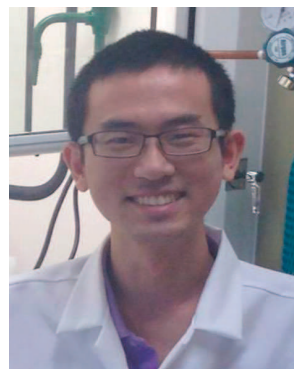
Dr Feng Li received his BS degree from Tianjin University, China, in 2006 and his PhD degree from the University of Alberta, Canada in 2013 under the direction of Professor X. Chris Le. His current research focuses on developing novel diagnostic tools for diverse biomarkers using dynamic DNA assemblies. He is also interested in constructing DNA assembled nanomaterials for diagnostic and medical applications.



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Kaixiang Zhang

Kaixiang Zhang is pursuing his PhD degree under the supervision of Professor Jinghong Li in Tsinghua University, China. From August 2012, he has worked in Professor Weian Zhao's laboratory as a visiting student at the University of California, Irvine. His research interests include DNA based biosensor and single cell analysis.

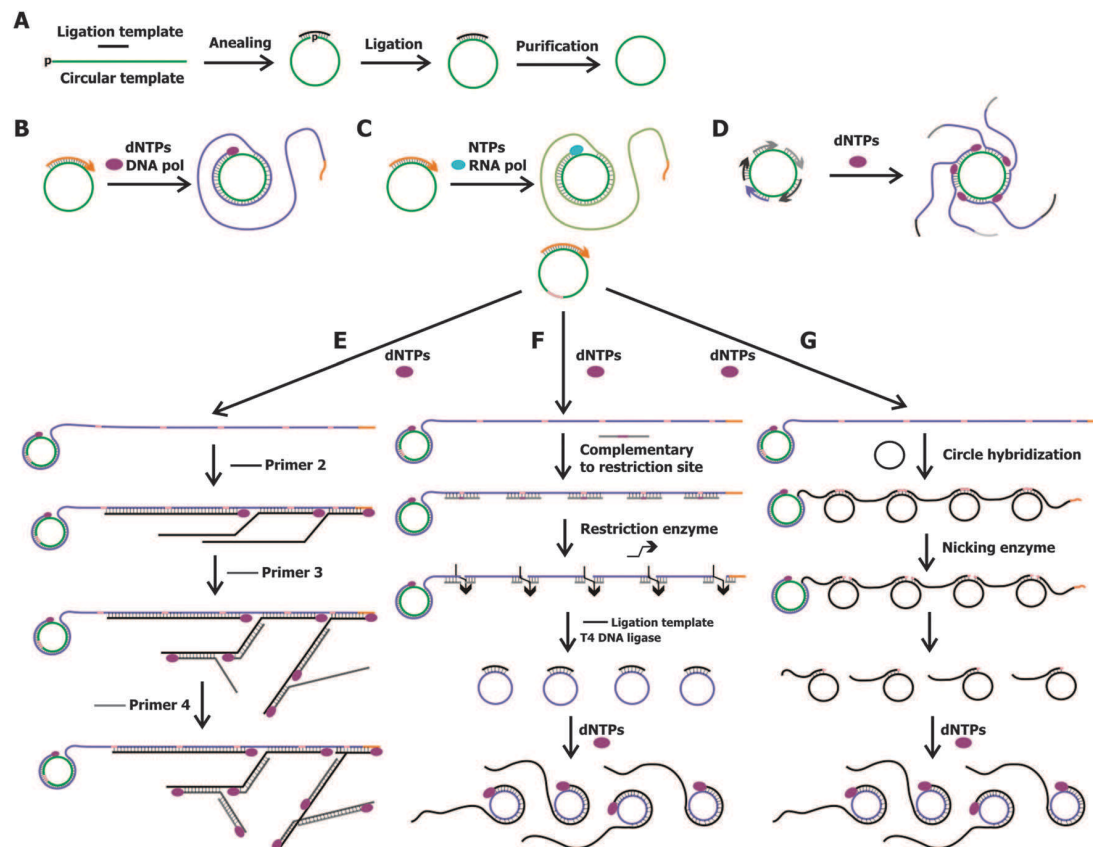


Fig. 1 Schematic illustration of the basic principles of RCA. (A) Making a DNA circle by template-mediated enzymatic ligation. (B) Linear RCA reaction to generate long ssDNA with a pre-made circle and target nucleic acids. (C) Linear RCA reaction to generate long RNA with a pre-made DNA circular template. (D) Multiprimed RCA to generate multiple copies of the RCA product from a single circle. (E) Exponential DNA amplification by hyper-branched RCA.^{91,93} (F) Exponential DNA amplification generating multiple circles from the RCA products.¹⁷ (G) Exponential DNA amplification by primer generation using nicking enzymes.⁵

a very complex circular DNA template with topological constraints, four-way junctions and multiple cross-overs.^{83,84} With respect to the RCA primer, both RNA and DNA, which are often the “target”

molecules one wants to detect, can serve this purpose. In fact, the target DNA and RNA can first be used as a ligation template to circularize a padlock probe that is used as the circular



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Weian Zhao

Dr Weian Zhao is an Assistant Professor at the Department of Pharmaceutical Sciences at University of California, Irvine. Dr Zhao completed his BSc and MSc degrees in Chemistry at Shandong University and obtained his PhD in Chemistry at McMaster University in 2008. During 2008–2011, Dr Zhao was a Human Frontier Science Program Postdoctoral Fellow at Harvard Medical School, Brigham and Women's Hospital and MIT. Dr Zhao's research aims to elucidate and control the fate of transplanted stem cells and to develop miniaturized devices for cancer diagnosis and point-of-care applications.

Table 1 Essential parameters that can be modulated in RCA

Parameters of RCA product	Rationale & methods
Length and valency	RCA reaction time
Sequence	Sequence of circular DNA template
Composition and modification	(1) DNA aptamers, DNazymes, spacer domains, and restriction enzyme sites: complementary sequences are encoded in the circular template. (2) Modifiers including fluorophores, biotin and amine can be introduced using modified dNTP during RCA reaction. (3) Functional moieties including fluorophores, biotin, antibodies, and nanoparticles can also be incorporated by hybridizing RCA products with complementary oligonucleotides tethered with those species. (4) Multiplexity and multifunctionality can be readily achieved through incorporation of multiple types of functional moieties as mentioned above. (5) Number of functional moieties on RCA product can be tuned by the length and valency of RCA product or by adjusting molar ratios of reagents; the distance between functional moieties can be adjusted by the length of spacer DNA domain.
Structure	Sequence of RCA product and “helper” complementary strands
Graft density on a surface	Molar ratio of primer and a “dilutor” molecule (e.g., polyethylene glycol (PEG))
Flexibility and rigidity	Sequence of RCA product and modulation between ssDNA and double-stranded (dsDNA) domains through hybridization of complementary strands.

template for RCA reaction. The circular DNA template (typically ~15–200 nt in length) can be synthesized enzymatically or chemically through the intramolecular ligation of phosphate and hydroxyl end groups.^{2,11,15,79,85} Most commonly, a circular DNA template is synthesized *via* template mediated enzymatic ligation (e.g., T4 DNA ligase) (Fig. 1A) or template-free ligation using special DNA ligases (*i.e.*, CircLigase).^{2,11,15,79,85,86} For synthesis of RNA products, a sequence that is complementary to T7 promoter sequence is added to the circle. A T7 promoter, which serves as the primer, is then annealed to the circular template. Transcription is then carried out by T7 RNA polymerase in the presence of nucleotide triphosphates (NTPs) instead of dNTPs (Fig. 1C).⁶ It has also been shown that RNA synthesis can be performed by RCA using a mini circular DNA template in the absence of primer and promoter using T7 and *Escherichia coli* RNA polymerases.^{87,88} By designing the circular template and primers, the length, sequence, composition, structure, and rigidity of the RCA product can be finely tuned, making RCA a highly versatile technique (summarized in Table 1).

3. Exponential RCA amplification

One of the powerful attributes of RCA is that circular templates can be designed so that the signal from a single binding event is amplified in an exponential manner (Fig. 1D–G). By employing multiple primers that hybridize with the same circle, multiple amplification events can be initiated producing multiple RCA products (“Multiprimed RCA” Fig. 1D).^{64,86,89} The number of primers that one circular template can accommodate depends on the length of the primers and the circle. Another exponential amplification method used in RCA is the so-called hyperbranched RCA (HRCA) (or ramification amplification) where the RCA product is used as the template for further amplification with the second and third sets of primers (Fig. 1E).^{90–93} Note that multiprimed and hyperbranched RCA (*i.e.*, combination of strategies in Fig. 1D and E) can be integrated to improve the sensitivity, especially when detecting targets in low abundance.⁶⁴ Moreover, Nilson and co-workers

have shown that a linear RCA product can be converted into multiple circles using restriction enzyme digestion followed by template mediated enzymatic ligation. These new circles can then be employed for further amplification using a second primer set (Fig. 1F).^{17,94} The restriction digestion, circularization and amplification in this “circle-to-circle amplification” process can be repeated for additional amplification. Finally, Komiyama and co-workers have demonstrated that RCA can be used to generate multiple primers by treating the RCA product with nicking enzymes upon hybridization with the second set of circles (Fig. 1G).^{5,95} The hybridized primer/circular template products obtained from this nicking enzyme reaction can be used directly for further RCA amplification.

4. Visualization and analysis of RCA product

The RCA process and product can be monitored and detected, respectively, using a variety of signal readout techniques. The most common way to analyze RCA product is gel electrophoresis.^{11,15,16} Additionally, the incorporation of fluorescent dyes to RCA products *via* fluorophore-conjugated dNTP during RCA (Fig. 2A) or hybridization of fluorophore-tethered complementary strands (Fig. 2B) permits easy visualization using fluorescence based techniques including fluorescence spectroscopy, microscopy, and flow cytometry.^{16,18,19,50,94,96,97} Similar to fluorescent probe hybridization, nanoparticles including gold nanoparticles (AuNPs), magnetic beads, and quantum dots can be assembled onto RCA products through complementary oligonucleotides to visualizing RCA products (Fig. 2B).^{22,98–102} Assembly of AuNP nanoparticles onto the RCA product allows colorimetric and spectroscopic visualization of the RCA product. Magnetic beads functionalized with complementary DNA sequences can be hybridized to the RCA product to produce a diffractometric signal for the detection of the RCA product.¹⁰³ An electrochemical signal can also be produced to achieve high sensitivity through quantum dot hybridization onto RCA products followed by dissolution.^{102,104}

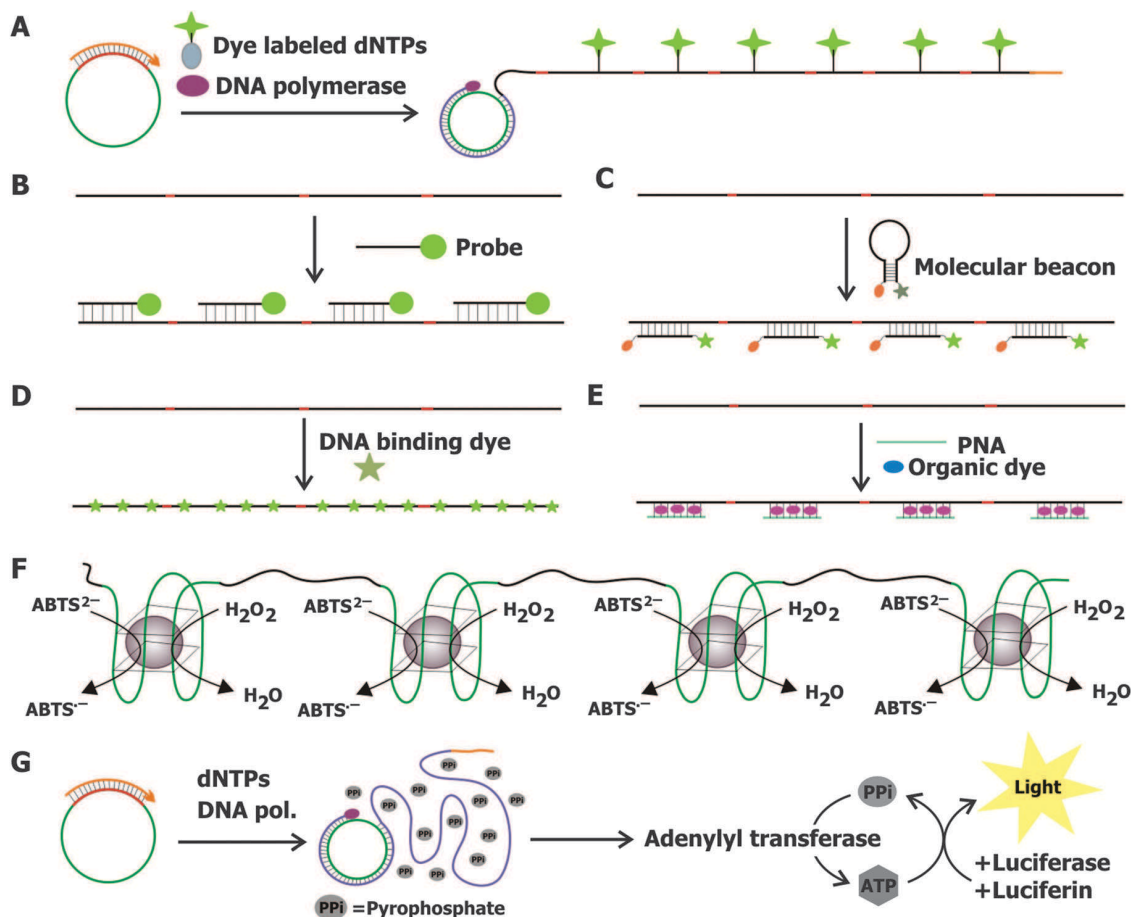


Fig. 2 Methods of detecting and analyzing RCA products. (A) Direct labeling of the RCA product by incorporating dye conjugated dNTPs during amplification reaction.¹⁹ (B) Detection of RCA product by probe hybridization. The probe can be a fluorescent molecule, gold nanoparticle or magnetic nanoparticle.^{16,50,96,98} (C) Fluorescent detection of RCA product by using molecular beacon.^{51,107} (D) Fluorescent detection of RCA product using DNA binding dye such as SYBR green.⁹ (E) Colorimetric detection of RCA product using DNA–PNA intercalating dye.¹¹ The free dye is blue in solution, but after binding to the duplex between RCA product and PNA, the dye turns purple. (F) RCA product as self reporter.¹² Each unit of the product is a catalyst and catalyzes a reaction to produce color. (G) Bioluminescence detection of RCA product through generation of light by using luciferase.^{40,110}

Another electrochemical-signal-generating method to detect RCA products includes the application of DNA-intercalating organic molecules such as methylene blue into the RCA product.⁴⁶ Molecular beacons (Fig. 2C) and DNA intercalating dyes (*e.g.*, SYBR green) (Fig. 2D) have also been widely used to detect RCA products and, importantly, to monitor the RCA reaction in real-time given their minimal background fluorescence in the absence of DNA products.^{9,51,82,105–109} In addition to fluorescent signals, some intercalating dyes, *e.g.*, 3,3'-diethylthiadicarbocyanine iodide (DiSC2(5)), can be used to generate a blue-to-purple color change when binding to a duplex between DNA and peptide nucleic acids (PNA) (Fig. 2E).¹¹ Such colorimetric signal changes allow for naked-eye detection of targets, which is particularly well suited for point-of-care diagnostic applications. Furthermore, chemiluminescence, generated by streptavidin-conjugated horseradish peroxidase (HRP) immobilized on the RCA product through biotin modified complementary DNA, has also been used to detect RCA products.^{12,13,110,111} Interestingly, HRP-mimicking DNzyme sequences that catalyze the oxidation

of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and generate a blue–green colorimetric signal can also be incorporated into RCA products (Fig. 2F).¹² This “dual-amplification system” (*i.e.*, RCA and DNzyme that has multiple turnovers) permits ultrasensitive colorimetric detection of the target molecule in real time. Finally, RCA products can also be detected through bioluminescence (Fig. 2G).^{40,112} In this case, the RCA reaction produces large amounts of pyrophosphates (ppi) that can be used as substrates by adenylyl transferase to produce ATP. The ATP is then used as a cofactor by firefly luciferase to generate a bioluminescent signal.

5. Applications of RCA in diagnostic and biosensing assays

RCA can amplify a single molecular binding event over a thousand fold, making it ideal for numerous applications that require ultrasensitive detection. The isothermal nature of RCA

provides new possibilities for point-of-care diagnostics compared to other techniques such as PCR that use sophisticated and expensive instruments (*e.g.*, a thermal cycler). Due to these attractive features, a variety of RCA-based platforms have been developed to detect a variety of targets including DNA, RNA, SNP, proteins, pathogens, and diseased cells. Here we organize

these technologies based on whether RCA is performed in the solution phase or on a surface.

5.1. Solution-based detection

In solution-based RCA, primers for the RCA reaction are present in solution, thus the resulting RCA products are homogeneously

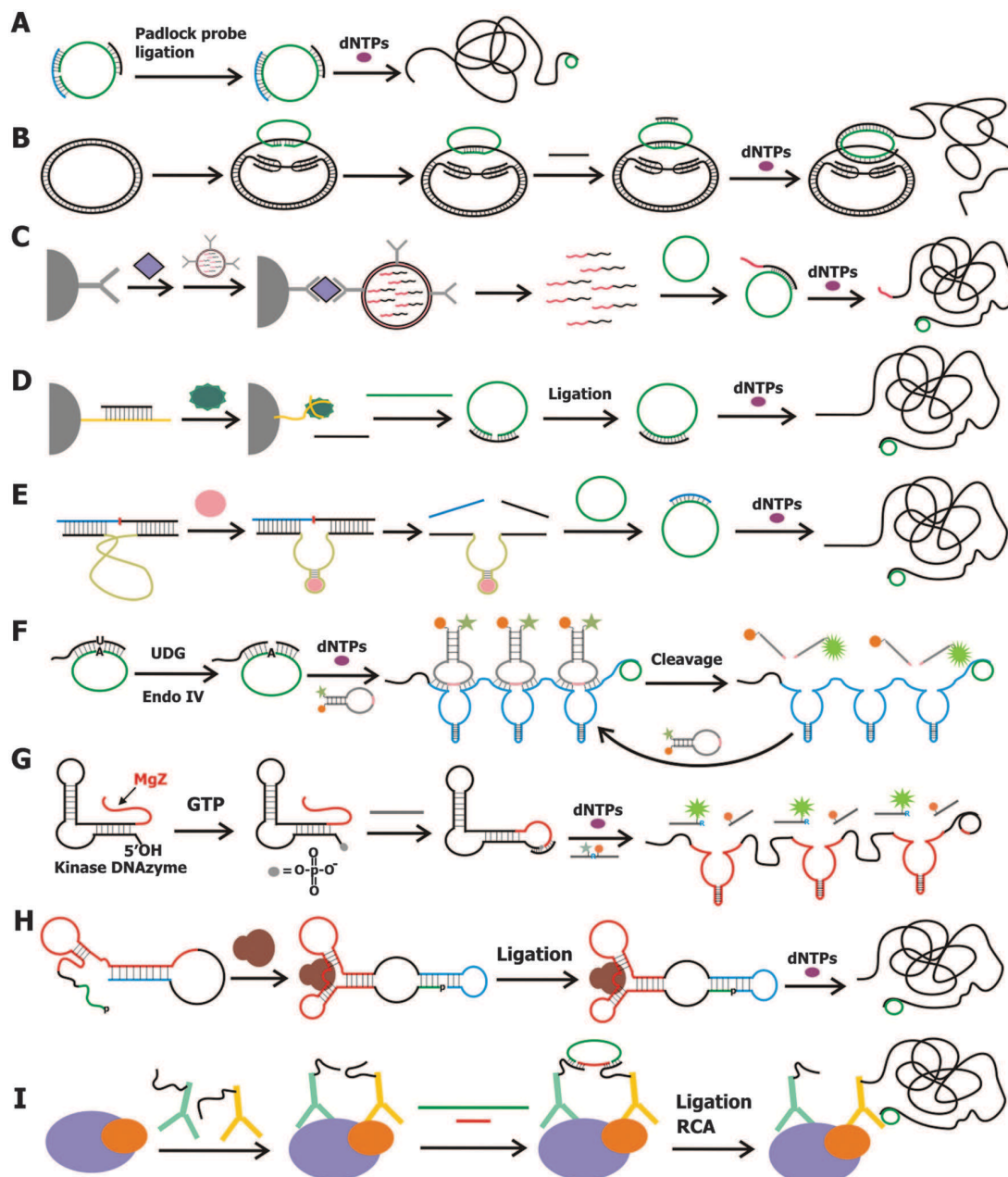


Fig. 3 Schematic illustration of some representative approaches of solution phase RCA detection. (A) A padlock probe based RCA. The padlock probe is circularized with the use of the target DNA or RNA as a ligation template.^{112–115} (B) RCA to detect genomic DNA by using PNA as duplex opener to circularize a padlock probe.^{126,127} (C) Capturing and releasing a large number of RCA primers through an antibody functionalized liposome. Multiple RCA primers are first captured on a solid support through antigen–antibody interaction. The primers are then released in the solution to take part in RCA reactions.¹²⁸ (D) Target detection by RCA using a structure switching aptamer.⁵¹ (E) Coupled RCA with RNA-cleaving allosteric DNAzyme to detect the target analyte.¹¹ (F) Detection of a DNA repair enzyme by producing an autocatalytic DNAzyme.¹³¹ (G) Detection of GTP using GTP-dependent kinase DNAzyme.¹³² (H) Binding-induced circularization of the aptamer to be used in RCA.¹³⁴ (I) Proximity-ligation-based RCA to detect target.^{135–137}

and dynamically free in the reaction solution (Fig. 3). Solution-based RCA has been widely adopted for the detection and analysis of nucleic acids, SNP, epigenetic modifications (*e.g.*, DNA methylation), proteins and small molecules.

5.1.1 Detection of nucleic acids and SNP. Ultrasensitive detection of specific DNA or RNA sequences and analysis of DNA variations at a single nucleotide level (*e.g.*, SNP) is of great interest for genetic research, diagnosis and personalized medicine. There are two common RCA methods used to detect nucleic acids. First, target nucleic acids can serve as the primer to trigger formation of RCA products through a pre-made circular DNA template (Fig. 1B). This simple, stand-alone RCA method has been used for homogeneous analysis of DNA and RNA where low-efficiency ligation steps are not required.^{32,33,36,113} In the second more widely used strategy, the target nucleic acids are used as a ligation template to join the ends of a circularizable DNA oligonucleotide ("padlock probe") followed by RCA using either the same target as the primer or by adding an external short DNA primer (Fig. 3A). To circularize the padlock probe to be used in the RCA process, it is critical that both ends of the probe are fully hybridized to the target nucleic acids, thus providing extremely high selectivity.^{66,67,114–117} Even a single mismatch in the ligation junction does not allow circularizing of the probe, which makes the padlock probe approach a powerful tool to detect SNPs and mutations.^{44,46,49,118,119} For example, Faruqi *et al.* has used this strategy for genotyping 10 SNPs in human genomic DNA in a high-throughput manner. Remarkably, they found that SNP scoring by ligation exhibited a 100 000 fold discrimination against probes mismatched to the SNP.¹²⁰ In addition to DNA and SNPs, the padlock probe approach has also been utilized to detect messenger RNA (mRNA),²⁹ microRNA (miRNA),^{105,116,121–123} and DNA methylations.^{38,39,124} To detect methylation, the target DNA sequence is first treated with bisulfite to convert the unmethylated cytosines into uracil leaving the methylated cytosines unaltered. A padlock DNA sequence is designed to be perfectly hybridized with the methylated target sequence. Circularization by enzymatic ligation and RCA is carried out as discussed earlier. If the sequence is unmethylated it does not hybridize with padlock probe and no circle is formed to take part in the RCA reaction. Thus only the positive RCA reaction is indicative of DNA methylation.^{39,124}

Whole genome amplification (WGA) is important for genome research such as sequencing to discover disease related genes. For this purpose, bias-free amplification is extremely critical to obtain a high quality amplicon with error free information. Furthermore extremely small amounts of genomic DNA limit successful sequencing. In many cases, PCR, due to the amplification bias to specific sequences and mutation during amplification cannot provide accurate sequencing results. RCA has proven to be highly useful in WGA using random hexamer primers.^{42,69,70,125} The hexamers randomly hybridize to the circular genomic sequence in multiple locations to produce multiple RCA products that further serve as templates to be amplified with the remaining free hexamers in the pool, thus providing large amounts of high quality DNA for sequencing and analysis.⁷⁰ Genotyping is also extremely valuable to determine an individual's genetic make-up

and alleles that have been inherited from parents. Nilson and colleagues have employed RCA for *in situ* genotyping in whole cells for both genomic DNA and mRNA. In the case of genomic DNA analysis, duplex DNA was first fragmented by restriction digestion, followed by exonucleolysis, to generate ssDNA sequences that circularized padlock probes for the RCA process.^{66,67} In the case of mRNA profiling, the mRNA was first reverse-transcribed to generate ssDNA that was then detected by padlock probe circularization and the RCA reaction.¹²⁶ Taking the advantage of 3'-exonucleolytic activity of Phi29 DNA polymerase, they have also shown that mRNA can be used as a template to circularize the padlock probe and, at the same time, the mRNA can be converted into a primer for RCA. Note that the 3'-exonucleolytic activity degrades the mRNA only in the single stranded region. When the nucleolytic action reaches the duplex region between the circularized padlock probe and mRNA, the polymerase starts adding deoxynucleotides to the mRNA sequence to generate the long ssDNA concatemer.¹²⁷ More recently, Smolina *et al.* have conducted RCA *in situ* in whole cells to detect X- and Y-specific sequences on human chromosomes. In their approach, they used a pair of PNA to locally open a target dsDNA site, allowing a padlock DNA probe to access the site and become ligated to form the circle to take part in the RCA reaction (Fig. 3B).^{128,129} A major advantage of using RCA for *in situ* genotyping in the above-mentioned examples is that the process results in strong, discrete signals localized to the target molecules, which permits easy visualization of their expression, location and distribution inside cells.

5.1.2 Detection of proteins and small molecules. The detection of proteins and small molecules with solution-phase RCA can also be achieved by target binding-induced generation of RCA primers or circular templates. For instance, Ou *et al.* reported a sandwich immunoassay where target antigen first captures an antibody functionalized liposome capsule that contains multiple copies of RCA primers (Fig. 3C). Lysis of the captured liposome releases the primers required for the subsequent RCA detection process.¹³⁰ In a similar immunoassay, AuNPs were used to carry hundreds of primers for on-nanoparticle RCA for ultrasensitive protein detection.¹³¹ Cascade signal amplification can also be achieved for sensitive protein detection using streptavidin conjugated antibodies that carry multiple RCA primers.¹³² Recently, a structure switching aptamer has been coupled with RCA to develop a sensitive method to detect cocaine (Fig. 3D). A cocaine aptamer hybridized with a short complementary sequence was first immobilized on a solid support. Addition of cocaine induced a conformational change in the aptamer domain to form a complex with cocaine and released the short complementary sequence which was used to circularize a padlock probe and then as the primer for an RCA reaction.⁵¹ Ali *et al.* reported an approach to detect ATP by combining the action of allosteric RNA-cleaving DNazyme and RCA (Fig. 3E).¹¹ An allosteric RNA-cleaving DNazyme is a DNazyme that can cleave a DNA-RNA chimeric substrate at a single ribonucleotide junction only in the presence of a specific target. In this example, the target ATP triggers DNazyme cleavage and the resulting 5' portion of the cleaved substrate serves as the primer for the

RCA reaction. Yu and colleagues have introduced an approach to monitor DNA repair enzyme activity *via* generating multiple autocatalytic DNAszymes to process fluorogenic substrates by RCA (Fig. 3F), given that DNA repair enzymes such as base excision repair enzymes (BER) play a vital role in biological events to maintain genome integrity. Using uracil–DNA glycosylase (UDG) as a model system, they have shown that the target DNA sequence containing a uracil nucleotide can be converted into an abasic site (AB) by UDG when hybridized with a circular DNA template. The AB site is then nicked by endonuclease IV (Endo IV) and used as a primer for the RCA reaction.¹³³ Li and co-workers have applied a similar strategy to convert a kinase DNAszyme into a sensor to detect guanosine triphosphate (Fig. 3G).¹³⁴ The DNAszyme phosphorylates at the 5'-hydroxyl group of a DNA substrate that is covalently attached to the DNAszyme. Another end of the DNAszyme contains a complementary sequence domain of an RNA-cleaving fluorogenic DNAszyme called MgZ.¹³⁵ The phosphorylation reaction of the DNAszyme in presence of GTP allows circularization of whole sequence to be used in the RCA reaction. During RCA reaction, the complementary domain of MgZ translates into multiple MgZs and cleaves a fluorogenic DNA–RNA chimeric substrate producing high fluorescence signal. RCA for protein detection can also be achieved using structure-switching DNA aptamers that can be circularized upon interaction with its target. For instance, Ellington and coworkers reported that the PDGF aptamer undergoes conformational change upon binding to PDGF to form a circularizable structure that can be ligated using T4 DNA ligase to form the RCA circular template (Fig. 3H).¹³⁶ Another common RCA-based protein detection system is the so-called “*in situ* proximity ligation (PL) strategy” where oligonucleotides tethered to antibodies against the two epitopes of the target proteins or protein complexes serve as the template to form circular DNA when bound in close proximity. The DNA circles can in turn serve as templates for localized RCA, allowing target proteins to be visualized and detected *in situ* in cell lines and clinical specimens (Fig. 3I).¹³⁷ Combining RCA with this PL strategy has also been employed to detect short RNA at the single cell level and cell surface markers.^{138,139}

Combining RCA with microfluidic devices provides additional advantages in detecting and analyzing biomolecules. For example, Nilsson and co-workers have integrated both padlock probe and proximity ligation based RCA with microfluidic devices for digital quantification of nucleic acids and proteins.^{140,141} They found that long RCA products from each molecular event yielded discrete DNA coils of approximately 1 μm size, each of which appeared as a bright object (~ 100 fold brighter than the free probes in solution) upon fluorescent probe hybridization. When pumped through microfluidic channels under a fluorescent detector the coils could be counted to obtain the number of biomolecules in the sample.

5.2. Solid-phase detection

Solid-phase RCA strategies where RCA is performed on a surface including glass, micro- or nano-bead, microwell plates, microfluidic devices or paper strips have also been developed

and are advantageous due to the easy separation of target from complex sample matrices, high throughput analysis capability, and potential uses in point-of-care settings (Fig. 4). For instance, target nucleic acids can be captured by surface-tethered DNA oligonucleotides and then serve as a template to ligate a primer for the surface-initiated RCA reaction (Fig. 4A). Ali, *et al.* has utilized this strategy for DNA detection on a paper strip.⁹⁶ This RCA primer ligation based approach has been used by other researchers to detect DNA mutation on magnetic beads.¹⁴² Smith, *et al.* used a similar strategy for large-scale SNP detection on a gold-coated glass substrate by combining surface-initiated RCA with an invasive cleavage reaction.¹⁴³ Specifically, an upstream oligonucleotide and a surface-tethered probe oligonucleotide were immobilized on the surface. Addition of the target DNA brought all three sequences together producing a small overlap between the probe and the upstream sequences (Fig. 4B) through Watson–Crick base-pairing. A flap endonuclease was then added to cleave the probe oligonucleotide at a specific nucleotide junction in the overlapped region producing a 5' phosphate group, which was then ligated with primer and used for RCA. In an alternative approach, a padlock probe was first hybridized to a surface-tethered capture DNA probe (Fig. 4C). The target DNA sequence served as the template to circularize the padlock probe and then the RCA reaction was initiated on the surface using the immobilized capture DNA probe as the primer.¹⁴⁴

Immunoassays especially ELISA are still one of the most commonly used bioanalytical tools in clinics and biomedical research. The RCA technique has been combined with ELISA to achieve higher sensitivity and reduce the lower detection limit. For instance, the target antigen immobilized on a solid support is treated with a RCA primer conjugated antibody (Fig. 4D) followed by an RCA reaction. This approach is also called immuno-RCA. Ward and co-workers demonstrated the first example of immuno-RCA for the quantification of IgE on a glass slide.¹⁰⁸ Since then, solid-phase-RCA strategies have been widely used as signal amplification components on antibody microarrays for multiplexed protein analysis.^{145–148} Similarly, a sandwich immuno-RCA has been adapted to achieve sensitive target detection. In this case the target antigen is first captured on a solid surface using an immobilized antibody (Fig. 4E). Next a secondary antibody conjugated to a RCA primer is applied to conduct the RCA reaction.^{7,103,149} As mentioned in Section 4, RCA products can be stained with complementary DNA-conjugated nanoparticles (*e.g.*, AuNP and quantum dot) and microbeads which provide additional means for signal detection and amplification in sandwich based solid-phase immune-RCA. For instance, Yin and co-workers developed an ultrasensitive protein assay (down to 16 molecules in a 100 μL sample) using RCA and anodic stripping voltammetric detection.¹⁰² In their study, the target protein (vascular endothelial growth factor) was first captured by an antibody-immobilized microplate and then complexed with a biotinylated antibody to form a sandwich immunocomplex. Through a streptavidin bridge, the biotinylated RCA primer was then attached to the microplate to initiate the RCA reaction. The resultant RCA product was then

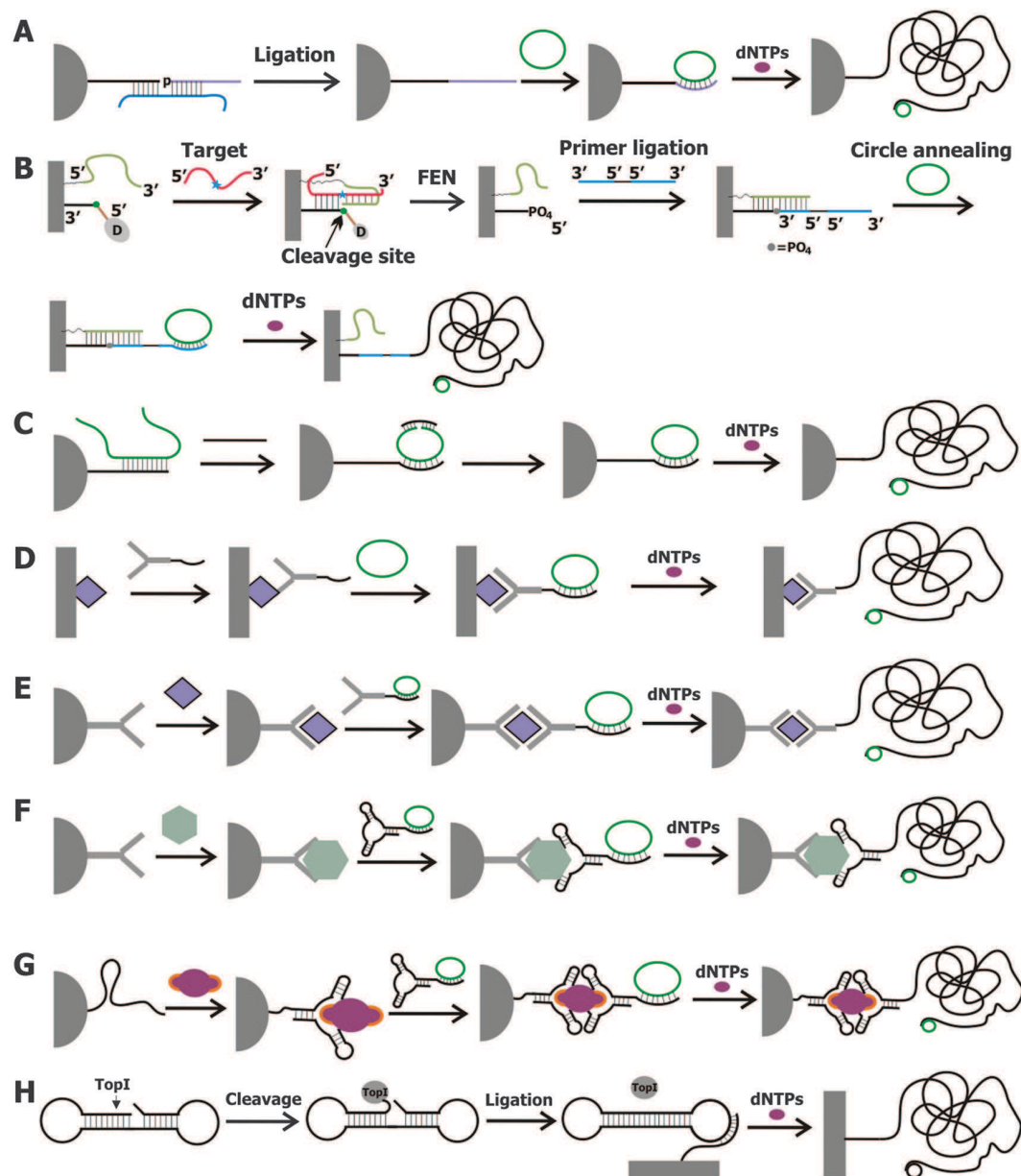


Fig. 4 Schematic illustration of some representative approaches of solid phase RCA detection. (A) RCA primer ligation on a solid support using the target as template.⁹⁴ (B) Surface invasive cleavage based RCA. A probe DNA (labeled with D for dabcy) and an upstream DNA (light green) are immobilized on a surface.¹⁴¹ The target binds to the probe and the upstream DNA with a small overlap with the probe DNA. A 5'-endonuclease, flap, cleaves the probe DNA producing 5'-phosphate which is ligated to a RCA primer to conduct the RCA. (C) Detecting the target on solid support using padlock probe.¹⁴² The padlock probe is first captured on a solid support which is then circularized in the presence of the target sequence followed by RCA. (D–F) Immuno-RCA on a solid support to detect antigens using antibodies or a combination of antibody and aptamer.^{100,106,149} (G) Aptamer based sandwich RCA.¹² (H) Generation of circles using naturally occurring repair enzymes (TopI).¹⁵⁴ TopI cleaves at three nucleotide upstream positions from the nick followed by sealing the gap to circularize the DNA. RCA is initiated with the primer immobilized on a solid support.

used as a template to assemble CdTe quantum dots which were finally dissolved and quantified by anodic stripping voltammetry. In another study, Savran and co-workers performed a sandwich-based RCA reaction on micropatterned glass slides. Magnetic microbeads were then assembled to RCA products to form diffraction gratings that generate distinct diffraction modes upon illumination with a laser.¹⁰³ Immuno-RCA using

antibody and RCA primers functionalized carbon nanotubes has also been applied to achieve ultrasensitive protein detection on a solid surface.¹⁵⁰ In some cases, antibodies can be replaced with high affinity DNA aptamers that can be conjugated to RCA primers through standard DNA synthesis and therefore eliminate the extra antibody–primer conjugation steps used in immuno-RCA (Fig. 4F and G).^{12,151} We have utilized this strategy for the

colorimetric detection of PDGF-BB on magnetic beads by incorporating HRP-mimicking DNazymes into the RCA product as described earlier (Fig. 4G).¹² Zhang and co-workers have applied a similar strategy to detect cancer cells as target.¹⁵²

Finally, RCA has also been used as a signal amplification tool to detect low abundance markers on the surface of cells (which technically is not a “solid” surface). For instance, Konry *et al.* employed cell-surface RCA assay to detect EpCAM on PC3 human prostate cancer cells.⁸ They first fixed target cells on a glass slide and then applied biotinylated anti-EpCAM antibodies. Biotinylated RCA primers were then immobilized with the anti-EpCAM antibodies on the cell surface *via* avidin linkages. RCA conducted in the presence of Cy3-dCTP permitted simple visualization of RCA amplicons on the cell.

In the same study described above, RCA was combined with droplet microfluidics to visualize efficiently the low abundance EpCAM biomarkers on the cell surface.⁸ They encapsulated the PC3 cells in picoliter size microdroplets in fluidic channels at a single cell level including the RCA reaction components. The RCA reaction with primer conjugated anti-EpCAM antibodies on the cell surface in an encapsulated microdroplet produced a localized bright fluorescent signal. Singh and co-workers have applied proximity ligation based RCA to detect microRNA (miRNA) within cells with the use of digoxigenin-conjugated locked nucleic acid (DIG-LNA) probe.¹⁵³ Antisense DIG-LNAs were hybridized with specific miRNA within permeabilized fixed cells in microfluidic channels. DNA oligonucleotides conjugated anti-DIG antibodies were then added to bind to DIG on the hybridized LNA sequences inside the cell followed by padlock probe circularization and RCA amplification. After staining the RCA product with fluorescent probes, the cells were detached from the microfluidic surface and analyzed by flow cytometry. This approach allowed efficient visualization of the miRNA expression level inside cells. Knudson and his group introduced an innovative approach to detect naturally-occurring enzymatic events in cells using RCA in microfluidics.^{154–156} They utilized the advantages of some naturally occurring DNA breaking and sealing enzymes such as Topo isomerase I (TopI). TopI enzyme catalyzes a transient DNA break followed by ligation to seal the gap. They utilized the advantage of the cleavage–ligation properties of TopI to generate DNA circles to be used in RCA reactions (Fig. 4H). Using this approach they have detected rare cells and parasites in biological samples. Furthermore they have also used DNA recombinase enzyme (flp recombinase) to generate circular templates to detect the activity of recombinase.¹⁵⁶ Using both enzymes they have formulated a multiplex assay for monitoring the relevant enzymatic activity.

6. Applications in nanobiotechnology and materials science

Nature has evolved numerous highly functional nanomachines comprised of proteins, nucleic acids, and other molecules to conduct complicated tasks in living systems. In particular, the highly specific Watson–Crick base pairing property and

engineering versatility (see Table 1) have made DNA a powerful and programmable building block to construct well-defined nano- and micro-scale architectures for a broad range of applications. RCA products are particularly attractive to construct multifunctional materials because they not only embrace all the above-mentioned advantages of nucleic acids but are also suited for periodic assembly, bridging the nano- and micro-scales due to their high molecular weight, and the development of multivalent synthetic ligands. Here we summarize recent advances in the use of RCA as a tool to construct nano- and micro-structures and materials for biosensing, drug delivery, therapeutics, tissue engineering, and studying and manipulating cell biology.

6.1. RCA products as periodic templates for nanoassembly

As mentioned earlier, the RCA product contains repeating sequence units that are complementary to the circular template which make it an ideal scaffold for periodic assembly of nanospecies at precise positions. For instance, Simmel and Mao's groups first demonstrated the use of RCA products as a 1D template for periodic assembly of complementary DNA functionalized AuNPs (Fig. 5A).^{21,77} We have also demonstrated that RCA can be conducted on gold nanoparticles (AuNP, 13 nm size) to form a 3D-like scaffold for further periodic assembly with a second set of AuNPs (Fig. 5B).¹⁵ Using a similar approach, Willner and co-workers assembled glucose oxidase (GOx)/AuNP seed (1.4 nm) conjugates on RCA products to grow metallic nanowires (Fig. 5C).⁸⁰ Specifically, GOx reacted with glucose to produce H₂O₂, which reduced AuCl₄[−] and enlarged the seed AuNPs to generate 1–5 μm long Au wires with a width of *ca.* 150 nm. In another work, Willner's group demonstrated that an RCA product containing aptamer sequences (*i.e.*, thrombin and lysozyme aptamers) could be used to assemble multiple proteins through aptamer–protein binding (Fig. 5D).¹⁵⁷ The assembly of nanoparticles on RCA templates provides interesting biophysical and optical phenomena which allow researchers to develop bioassays. In particular, the aggregation of AuNPs leads to a red-to-blue color change due to surface plasmon resonance which has been used to develop colorimetric assays.^{158,159} Recently, Li and colleagues have shown that AuNPs (13 nm size) assembled onto RCA products formed globular nanostructures in the presence of salt which they named DNA nano-islands (Fig. 5E).⁹⁸ Interestingly, AuNPs are separated by a specific distance from each other in the assembled complexes which retained a red color. This observation led them to develop a colorimetric bioassay for ATP using an RNA-cleaving allosteric DNzyme. As described in Section 5.1.2, the long RCA product was prepared using the 5'-portion of the cleaved DNA–RNA chimeric substrate as primer after the ATP-mediated cleavage reaction followed by the assembly of the AuNP conjugated complementary oligonucleotides. Addition of MgCl₂ induced formation of the RCA–AuNP nano-islands.

6.2. RCA-based nanostructures

DNA nanotubes have emerged as promising tools for a variety of biological and materials applications, especially, for drug delivery, because of their exceptional control over shape,

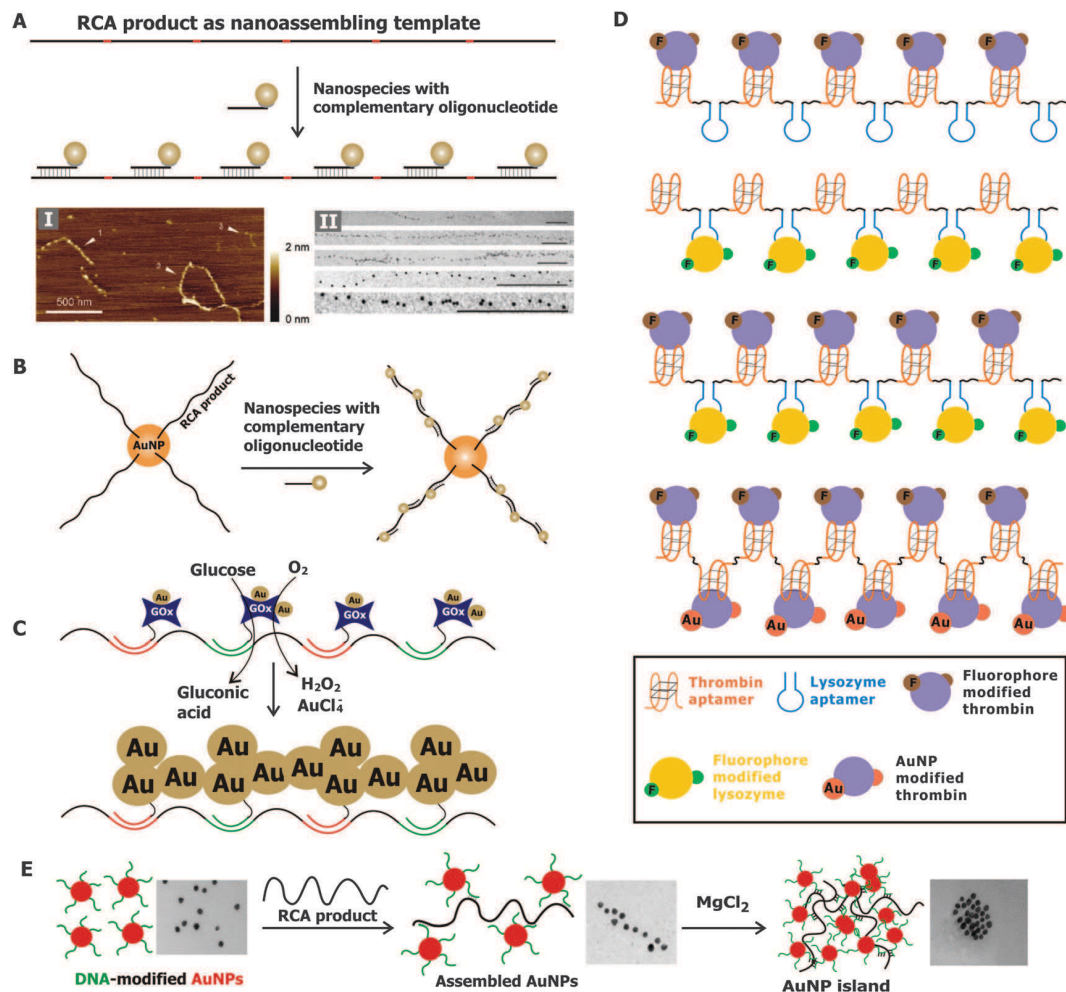


Fig. 5 RCA as templates for periodic assembly of nanospecies or proteins, synthesis of nanowires and formation of nano-islands. (A) 1D assembly of complementary DNA-probe functionalized AuNPs (I, II are AFM and TEM images of the AuNP assembled RCA product).^{21,77} (B) RCA on AuNP and 2D assembly of smaller particles on the RCA product.¹⁵ (C) Synthesis of gold nanowire on RCA template by periodic assembly of AuNP conjugated enzymes.⁸⁰ (D) Assembly of fluorophore and AuNP labeled enzymes (thrombin and lysozyme, shown in the inset).¹⁵⁵ (E) Formation of DNA-AuNPs nanoislands using RCA product.⁹⁸ (A) was reprinted from ref. 21 and 77, with permission.

length, rigidity and porosity and their potential for encapsulation and release of drugs in response to specific external stimuli. Sleiman and co-workers have developed a new strategy for the construction of DNA nanotubes using RCA products as the backbone (Fig. 6A).^{73–76} In a series of works, they produced DNA nanotubes that were assembled using long, continuous RCA product scaffolds, triangular “rungs” (made from a closed triangle DNA structure carrying three complementary strands with overhangs at each end), stimuli responsive block copolymers and a set of linking strands (Fig. 6A).^{73–76} By labeling with fluorescent dyes, they found that these nanotubes were more nuclease-resistant, capable of entering cells with significantly increased efficiency over dsDNA and amenable to encapsulation and release functions (Fig. 6A, I–IV).⁷³ Their works demonstrate that the assembly of special hybrid materials such as stimuli responsive block copolymer particles (including polyethylene glycol and polystyrene) on the above-mentioned

DNA nanotubes using simple DNA hybridization can be sequence-specific and longitudinally positioned on robust DNA nanotubes.⁷⁴ Importantly, block copolymer particles can be dissociated from DNA nanotubes when a complementary DNA is introduced through strand displacement mechanism.

Recently, Fan and co-workers have reported the construction of different kinds of DNA structures including 1D nanowires, and 2D nanoplates and nanoribbons using an RCA product as a backbone with the help of only a few staple strands (Fig. 6B).⁷² Interestingly, they found that the size, structure and configuration of these nanostructures can be tuned by the length of the RCA circular template, the concentration of the RCA products and its molar ratio to the staple strands. Using the same concept, they further demonstrated that it is feasible to construct more complex, multi-layer 3D nanoribbons (Fig. 6C).¹⁶⁰ Cellular uptake experiments revealed that these multi-layer nanoribbons exhibited significantly higher cell permeability

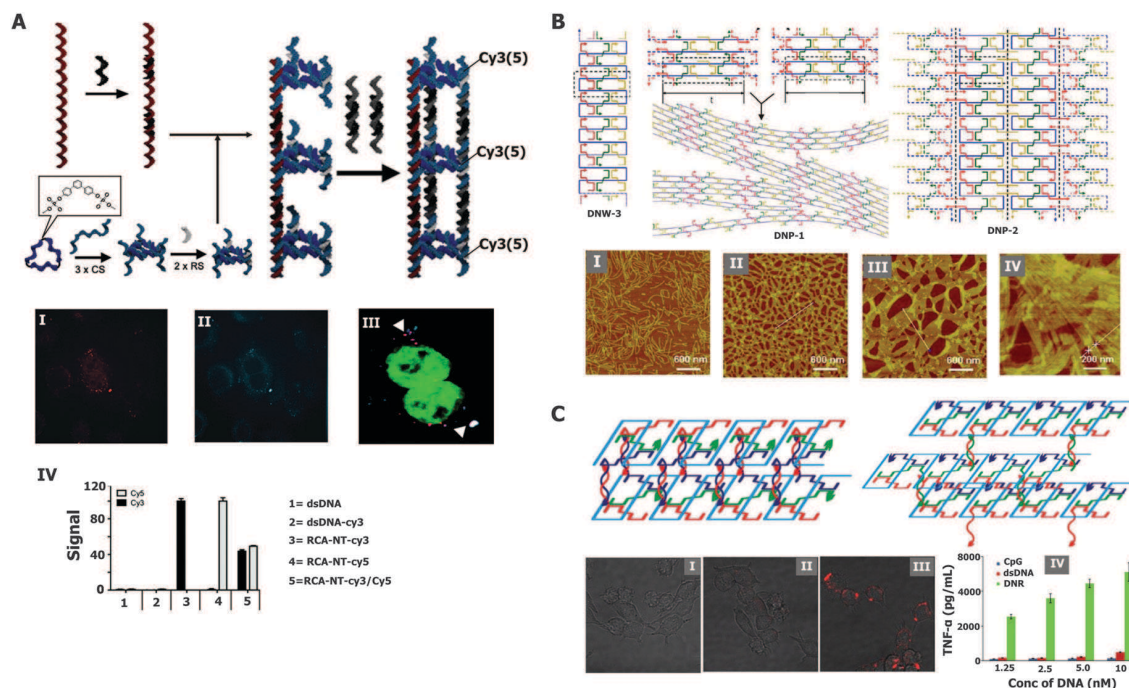


Fig. 6 Construction of DNA nanostructures using RCA. (A) DNA nanotube assembled from RCA produced long ssDNA.⁷³ DNA nanorings are assembled on the RCA product, generating a DNA tube. (AI, II, III) Confocal images of the HeLa cells after treating with Cy3, Cy5, and mixed Cy3–Cy5 conjugated nanotubes showing their cell internalization ability. (A IV) Comparison of cell internalization of the RCA nanotubes with the dsDNA alone. (B) DNA nanoplates, nanoribbons made with RCA product and few staple DNA sequences.⁷² B I, II, III are representative AFM images of the nanoplates and nanoribbons at different stages of growth. (C) Multi-layer nanoribbons with study of their cell internalization (I, II, III) and drug delivery (IV).¹⁶⁰ (A)–(C) were adapted and reproduced from ref. 73, 72 and 160, respectively, with permission.

compared to ssDNA and dsDNA (Fig. 6C, I–III).^{160,161} As discussed earlier, an important advantage of these DNA nanostructures is that they can be easily decorated with functional moieties including sensing probes, nanoparticles and drugs. Using CpG oligonucleotide as a model drug, Fan and colleagues demonstrated that CpG oligonucleotides could easily be incorporated into their nanoribbons through DNA hybridization. Significantly, DNA nanostructures modified with multiple CpG motifs exhibited a higher immunostimulatory effect, indicated by production of high amount of the inflammatory cytokine tumor necrotic factor alpha, than CpG oligonucleotide alone and CpG conjugated to a dsDNA domain that was used to assemble CpG on a DNA nanoribbon (Fig. 6C, IV).¹⁶⁰

6.3. RCA product as multivalent synthetic ligands

Multivalency is a key principle in many biological interactions in nature and particularly multivalent interactions between cell surface receptors and their ligands are critical to enhance functional binding affinity and selectivity and to modulate biological signaling through ligand-induced receptor clustering. Inspired by nature, chemists and material scientists have attempted to construct multivalent synthetic ligands with architectures including linear polymers, nanoparticles, and dendrimers to interrogate complex biological processes and develop therapeutics. Unfortunately, current methods to prepare multivalent synthetic ligands are complex and often involve

chemistries that are not easily amenable to attachment and spatial organization of receptor-binding moieties on these scaffolds.^{162–168} We have recently exploited RCA to produce multivalent ligands to capture cancer cells the blood as a cancer diagnostic and for targeted drug delivery to cancer.⁹ Capture and isolation of rare circulating tumor cells (CTCs) and residual cancer cells in human blood after treatment is an emerging approach for cancer diagnosis and prognosis, monitoring treatment and studying cancer biology. We developed a platform approach to capture and isolate circulating cells using a 3D DNA network comprising repeating adhesive cancer-binding aptamer domains made by RCA on a microfluidic device (Fig. 7A, I).⁹ These long DNA molecules extend over tens of micrometers from a 2D surface into 3D space, therefore maximizing the contact between the capturing aptamers and the flowing cells. Using an aptamer that binds to the protein tyrosine kinase-7 (PTK7) that is overexpressed on many human cancer cells as a model system, we demonstrated that the 3D DNA network significantly enhances the capture efficiency of lymphoblast CCRF-CEM cells over monovalent aptamers and antibodies, yet maintains a high purity of the captured cells (Fig. 7A, II and III). Our results suggested that long DNA with multivalent aptamers binds to target cells with a higher efficiency, which is likely due to a combination of higher binding avidity (multivalency), a higher probability of association (large size and 3D conformation), and unique deformable and elastic structures.

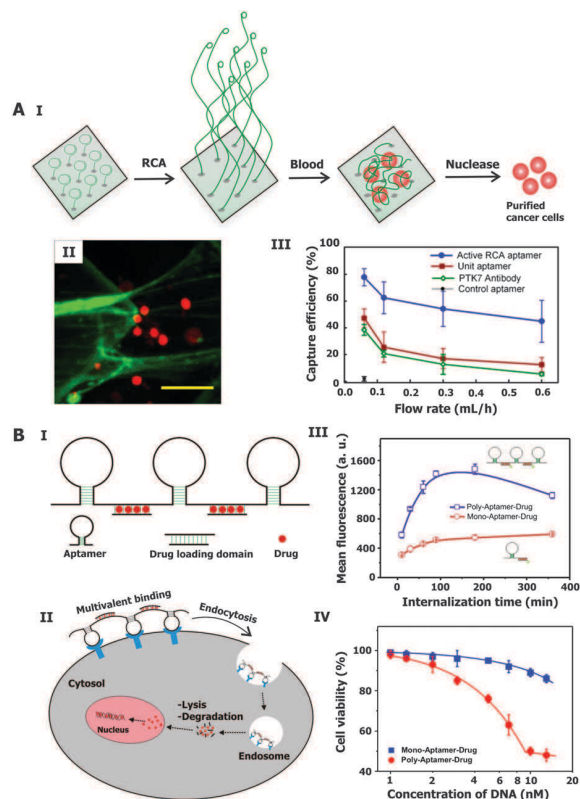


Fig. 7 Polyvalent ligand produced by RCA for cancer cell isolation and targeted drug delivery. (A) Generation of a long RCA product containing multiple aptamer units in a microfluidic channel and capturing and releasing cells. (AII) Fluorescent image of the RCA product (green) and captured CCRF-CEM cells (red) (scale bar 20 μm). (AIII) Comparison of cell capturing efficiency of the polyvalent aptamers with unit aptamers and antibodies under flow conditions. (B) Schematic design of polyvalent aptamers with loaded drugs. (BII) Schematic illustration of targeted drug delivery using the polyvalent aptamer through endocytosis and release of drugs inside the cell. (BIII, IV) Comparison of cell internalization and cell killing ability between polyvalent and monovalent aptamers. Adapted from ref. 9 and 10 with permission.

Additionally, captured cells can be released from the device using a minimally invasive process by simple nuclease treatment which allows one to investigate further the biology of these circulating cancer cells. More recently, we have developed multivalent therapeutics that are composed of multiple aptamer units synthesized by RCA and physically intercalated chemotherapy agents for specific and efficient cancer targeting (Fig. 7B).¹⁰ Using the same PTK-7 binding aptamer and CCRF-CEM cells as we described the above as a proof-of-principle; we have shown that the multivalent aptamers loaded with doxorubicin exhibited significantly more effectiveness than the monovalent counterpart in targeting and killing target cells due to enhanced binding affinity (~ 40 fold greater) and cell internalization *via* multivalent effects (Fig. 7B, III and IV). We reason that this new class of multivalent drugs will have great potential to address the poor efficacy and off-target systemic toxicity associated with current chemotherapeutic approaches. Interestingly, crosslinking of multiple cancer cells by the multivalent aptamer system could

be observed in our studies. Therefore, we rationalize that RCA may become a tool to assemble multiple cells to form tissue constructs for tissue engineering applications.

6.4. RCA-based hydrogels and DNA “glues”

The large size of the RCA product makes it a well suited building block to bridge the gap between the nano-, micro- and even macroscales to construct novel metamaterials and complex structures. Recently Luo and co-workers have reported a fascinating DNA hydrogel system made of 3D entangled RCA products.⁸² Remarkably, these new metamaterials possess unique mechanical meta-properties: they behave either liquid-like or solid-like depending on the physical environment (Fig. 8A–D). For example, the gel remains in solid-like form in water while flowing freely in a “liquid” form when it is taken out of water. The liquid form of the hydrogel can adopt different shapes of the container, and interestingly, it always returns

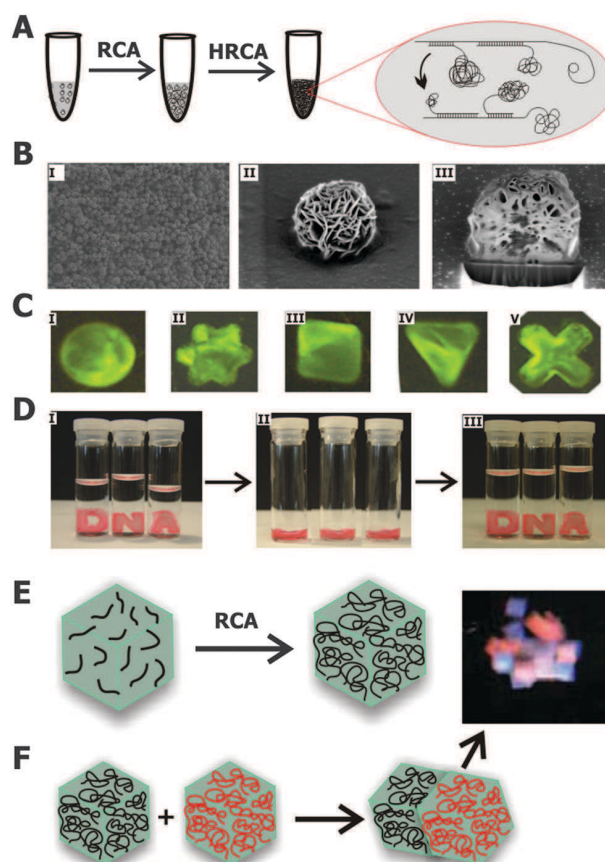


Fig. 8 DNA hydrogel as metamaterial and giant glue made by RCA. (A) Preparation of DNA hydrogel as metamaterial using RCA. (B) SEM images of the RCA hydrogel. (C, D) Tests of the metamaterial-like behavior of the RCA hydrogels. The hydrogel itself without water behaves as liquid and takes the shape of the mold where it is kept. In water it behaves as solid with a specific structure and upon removal of water the structure collapses and turns to the liquid phase. (E) Formation of giant DNA glue by RCA on RCA-primer grafted hydrogels. (F) Assembly of multiple hydrogels through sequence specific hybridization to product multiple-hydrogel objects. Adapted and reproduced from ref. 81 and 82, with permission.

rapidly to its original shape when placed back in water, regardless of how many different shapes it had adopted in the liquid-like state (Fig. 8A–D). Specifically, the DNA hydrogel is formed due to linker-free, DNA entanglement when an RCA reaction is performed and followed by multi-primed chain amplification (“MCA”, as described in Fig. 1E). They found that the hierarchical structure of the DNA hydrogel, which is attributed to the above-mentioned mechanical properties, can be tuned by changing separately the reaction time of RCA and MCA. Luo and colleagues hypothesized that the unique meta-properties of the DNA hydrogel may find applications in drug release, cell therapy, electric switches and flexible circuits.

More recently, Qi *et al.* have demonstrated that RCA can be employed to produce a “giant DNA glue” to assemble PEG hydrogels to form micro- and macroscale complex structures in a programmable manner. In their work they first prepared DNA primer-modified PEG hydrogels on which RCA was performed to generate long DNA glues (Fig. 8E).⁸¹ They included complementary sequences in the RCA product so that different hydrogels can be assembled through sequence specific hybridization. Mixing the hydrogels in a tube under rotation produced multi-hydrogel objects with sizes ranging from 30 micrometers to a millimeter (Fig. 8F). RCA has also been employed to produce a siRNA sponge on the microscale size for effective siRNA delivery and treatment. For instance, Hammond and colleagues have conducted an RCA reaction using a T7 promoter as the primer and T7 RNA polymerase to produce RNA products (Fig. 1C) which can self assemble to form a microscale RNA sponge for effective siRNA delivery.⁶

7. Summary and outlook

As a simple, efficient and isothermal nucleic acid amplification tool, RCA has now become a powerful tool in the development of bioassays and diagnostics in research, clinical and point-of-care settings. In particular, the integration of RCA with functional nucleic acids including aptamers and DNazymes, and with other assay platforms including PCR, ELISA, microfluidics, SPR (surface plasmon resonance) and nanoparticles presents new opportunities for ultrasensitive detection of a variety of targets including nucleic acids, proteins, small molecules, virus and cells.^{8,9,48,60,103,130,145,153,155,169–174} From a materials science perspective, the engineering versatility (Table 1) of RCA makes it an exciting tool to prepare DNA building blocks to construct highly-ordered nanostructures and new materials that may find real-life applications in drug delivery and controlled release, biosensing and imaging, and bioelectronics.^{2,6,73,82,98,160,161} Furthermore, RCA-based multivalent synthetic ligands that incorporate a number of variables which would otherwise be difficult to achieve in other multivalent ligand systems, including the number, the density, the type, and the spatial organization of ligands represent a new chemical biology tool to elucidate and modulate the function of cell surface receptors and to develop next generation therapeutics.^{9,10} In spite of many advantages, some challenges of the

RCA system for practical applications remain to be overcome. First, the preparation of high-purity circular templates in large quantity can be a practical challenge. For instance, in the enzymatic ligation approach, in addition to circular DNA, linear multimeric by-products can sometimes be formed.⁸⁵ This issue could be partly addressed by using a low concentration of DNA in the ligation reaction. The circular DNA products can then be purified from linear by-products by gel electrophoresis or by exonucleases treatment that only degrades non-circularized linear DNA molecules.¹² Additionally, the enzymatic ligation process is known to be efficient for relatively large DNA substrates, but may not be suitable for making small DNA circles (<30 nt), presumably due to inadequate enzyme binding sites and/or the strain induced upon the ring closure of short oligonucleotide.² This shortcoming however can be addressed by chemical cyclization of DNA oligonucleotides that can generate circular DNA molecules in reasonably good yield (up to 85%) for both small (<14 nt) and large circular templates (>15 nt).^{2,85} Additionally, other challenges of the RCA system for practical applications include mass production, purification, and storage as long RCA products tend to aggregate due to nonspecific inter- and intra-molecular crosslinking. Furthermore, nonspecific binding can occur when RCA products are used for cell targeting and drug delivery due to their large molecular weight. In our experience, these issues can be minimized by fine-tuning the parameters listed in Table 1 including RCA product length, sequence, composition and rigidity. For instance, we found that incorporation of a polyT (but not a random sequence) spacer between aptamer domains in the RCA product could reduce the nonspecific interactions between the multivalent aptamer system to cancer cells.⁹ Finally, computer-aided methods (*e.g.*, cadnano) can be used to design RCA sequences and staple strands to construct predictable DNA nanostructures to minimize unwanted non-specific interactions.

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